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The primary goals set forth in this research project are to establish optimal systems for the detection of both viral antigen and genomic RNA, and to use these detection systems to investigate viral pathogenesis in an animal model (guinea pig). The Pichinde virus was used as the prototype since it is a non-human pathogen.

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ANTIGEN AND GENOME DETECTION OF ARENAVIRUS, BUNYAVIRUS, AND FILOVIRUS INFECTIONS

Contract DAMD 17-88-C-8149

Midterm Report(September 1988-March 1990)

Introduction

The primary goals set forth in this research project are to establish optimal systems for the detection of both viral antigen and genomic RNA, and to use these detection systems to investigate viral pathogenesis in an animal model (guinea pig). The Pichinde virus was used as the prototype since it is a non-human pathogen. The following midterm report covers the progress for the period September 12, 1988 through March 12, 1990.

Antigen Detection

The initial phase of this work involved immunofluorescent studies on acetone-fixed Vero cells that were infected at various times after infection by Pichinde virus, using monoclonal and polyclonal anti-Pichinde antibodies. Checkerboard titrations of the primary antibody and corresponding fluorescein-labelled secondary antibodies identified the optimal dilutions for both. The P903 monoclonal antibody dilution was established at 1:400, which yielded specific staining for Pichinde antigen when followed by fluoresceinated goat anti-mouse IgG. The polyclonal hamster anti-Pichinde

antibody dilution was set at 1:200 when followed by fluoresceinated goat anti-hamster IgG. Background staining was minimized by using acid cleaned slides and adequate washing in PBS following antibody incubations. The use of TES (3-aminopropyltriethoxysilane) treated slides prevented cells from being detached during the different staining procedures.

The next step was to use the above concentrations of antibody along with enzyme-labelled (alkaline phosphatase-conjugated and peroxidase) secondary antibodies to establish an immunocytochemical protocol for use on infected cell cultures. Pichinde-infected and uninfected Vero cells were spotted on pretreated slides and fixed in each of the following fixatives for 5 minutes:

Acetone

10% Neutral Buffered Formalin

4% Paraformaldehyde

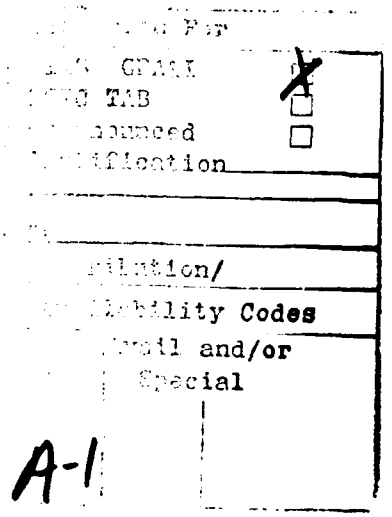
Omni

IEM

MBC

Zinc Formalin

B-5



With the exception of acetone, cells fixed with the other fixatives, all of which have cross-linking characteristics, required proteolytic treatment prior to incubation in the primary antibody to demonstrate positive staining of viral antigen.

Alkaline Phosphatase Detection

Acetone-fixed cells were incubated in P903 monoclonal antibody (1:400) overnight at 4oC, washed in PBS, and incubated in biotinylated rabbit anti-mouse IgG (1:1000) for 30 minutes at room temperature. Following washing in PBS, Streptavidin-alkaline phosphatase (1:250) was added for 10 minutes at room temperature, washed free of excess enzyme, and developed with Fast RedTR Salt. Infected cells revealed a red,cytoplasmic staining pattern whereas uninfected cells did not. Controls included omission of the primary antibody, substitution of the primary antibody with non-immune mouse serum, and replacing the primary antibody with an unrelated monoclonal antibody. All controls were consistently negative.

Proteolytic treatment of cells fixed in the cross-linking fixatives utilized Proteinase K at 5 ug/mL in

Tris buffer at 37C. Positive staining was observed using all fixatives with no apparent advantage cf one over the other. However, B-5 had the disadvantage of requiring extended digestion.

Immunoperoxidase Detection

As an alternative to alkaline phosphatase, experiments were conducted using peroxidase conjugated secondary antibodies followed by DAB (3,3-diaminobenzidine) chromagen which gives a brown reaction product. Endogenous peroxidase activity was blocked with hydrogen peroxide treatment prior to application of the primary antibody. Pichinde virus infected Vero cells stained with peroxidase-labelled secondary antibody had the same staining pattern as seen with alkaline phosphatase labelled secondary antibody. Proteolytic treatment was again required, except for acetone fixed cells.

The use of peroxidase conjugated antibodies on tissue sections during the next phase of the project also alleviated the problem of background staining due to endogenous biotin stores in the liver.

Pichinde Infected Tissues

We then applied the experimental data gained from the cell culture experiments to tissue sections.

Immunofluorescence experiments were performed on frozen, acetone fixed tissues. Because the various fixatives used in the cell culture experiments did not provide a signficant staining advantage, 10% Neutral Buffered Formalin was chosen as the fixative of choice because it is widely used as the routine fixative of choice in most pathology laboratories.

Immunofluorescence staining of frozen guinea pig tissues obtained from day 11 Pichinde virus infection revealed the same starburst, cytoplasmic staining pattern as seen in the infected Vero cells. Positive staining was particularly intense in lymphoid tissues, as well as lung and liver. Controls were consistently negative.

Immunohistochemical protocols for evaluating Pichinde virus infections were established and refined using alkaline phosphatase and peroxidase labelled secondary antibodies. Due to biotin stores in the liver, and endogenous alkaline phophatase in the kidney and gut, which gave increased background when using alkaline

phosphatase as the reporter molecule, despite steps taken to quench their activity, it was decided to concentrate on immunoperoxidase techniques. Initially, peroxidase staining provided a weaker signal than alkaline phosphatase. However, addition of NiCl2 to the substrate buffer markedly enchanced signal strength. The DAB chromogen, in the presence of NiCl2, yields a crisp, blue-black, insoluble product that provides a permanent marker.

In collaboration with the investigators at the University of Arizona, we obtained tissues from animals sacrificed at serial time points over the course of Pichinde viral infection of susceptible guinea pigs. These tissues were then examined for the presence of viral antigen and quantified on a +/- scale as depicted below (+ =weakly positive, ++++ =strongly positive).

	Control	Day 7	Day 11	Day 13	Day 17	Day 19	Day 23
LIVER	-	+	+++	+++	++	+	+
SPLEEN	-	+	+++	+++	+++	++	+
PANCREAS	-					+	++
KIDNEY	-	-	+	+	++	+	+
ADRENAL	-	+	++	+++	+++	++	++
HEART	-	-		+	++	+	
LUNG	-	+	++	+++	++++		+++
STOMACH	-			++	++	+	+
SMALL INT.	-	-	++	+++	++	+	+
LARGE INT.	-	+		+++	++	+	+

Double labelling immunofluorescence experiments, using both anti-Pichinde antibody and a murine monoclonal antibody, MR-1, specific for guinea pig histiocytes (macrophages) showed that the majority of cells in the lamina propria of the gastrointestinal tract, exhibiting viral antigen also reacted with the MR-1 antibody. This relationship is being examined in other tissues.

GENOME DETECTION

Viral genome detection has proven to be more difficult than anticipated, perhaps because the adapted Pichinde virus genome used to infected guinea pigs is not completely homologous with the prototype virus, from which the Pichinde cDNA clones were established. cDNA clones used by Auperin et al. to sequence the Pichinde viral genome were obtained as a gift from CDC. The A3-89 clone, which codes for the viral nucleocapsid protein, was chosen for nucleic acid probe preparations.

Northern blot analysis of mRNA from Pichinde infected Vero cells revealed strong positive bands when using ^{32}P labelled A3-89 cDNA probes. mRNA from uninfected Vero cells did not hybridize with the probe.

Multiple experiments were conducted on infected Vero cells as well as frozen, paraformaldehyde and formalin-

fixed infected tissue. A variety of probes were generated via nick translation, random priming. cDNA probes were tested using ^{35}S, beta-galactosidase, peroxidase, biotin, and digoxigenin as labels. Results with infected tissues were inconsistent with all cDNA tested despite attempts to satisfy the many parameters associated with in situ hybridization. These parameters include, but are not limited to:

1. Probe stabilization
2. Target stabilization
3. Probe concentration
4. Hybridization buffer content
5. Proteolytic treatment to facilitate probe entry (permeation)
6. Optimal temperature of hybridization
7. Length of hybridization
8. Stringency of posthybridization washes
9. RNase treatment
10. Autoradiography developement of isotopic probes
11. Chromogenic developement of non-isotopic probes

We have recently been focusing on RNA probes due to their increased specificity and thermal stability. Using the Riboprobe system from Promega, ^{35}S-UTP labelled RNA probes were generated by random priming. Concurrently,

Digoxigenin-UTP labelled RNA probes were also synthesized (Boehringer Mannheim) and are currently being tested in combination with anti-digoxigenin peroxidase labelled antibodies to intensify the signal. Silver amplification of the peroxidase/DAB complex should solve the previous problems of weak signal and provide a successful non-isotopic in situ hybridization protocol.

The increased background seen in previous ^{35}S RNA probe experiments appears to have been resolved by increasing the temperature of hybridization and the post-hybridization washes.

INTERPRETATION OF FINDINGS

Immunohistochemical findings on serially harvested infections are consistent with the interpretation that extensive primary infection with Pichinde virus in guinea pigs occurs in or about the lamina propria of the small intestine with extension throughout the gastrointestinal tract. The intestinal infection may cause cachexia and/or malabsorptive states which secondarily results in diffuse fatty change and focal necrosis of the liver (as previously noted). There is also deposition of Pichinde viral antigen in spleen and liver presumably due to either susceptibility of fixed tissue histocytes to infection and/or general phagocytic properties of of these those organs. The adrenal and the lungs are also

targets for Pichinde virus. Thus the cause of death in infected guinea pigs may be multi-organ failure with impairment of energy metablism, electrolyte balance, and acid-base status (eg, pulmonary effect on blood gases).

Future work will be focused on identifying sites of active viral replication (eg. with in situ hybridization using positive and negative strand-specific probes) to differentiate Pichinde virus infection of histocytes from accumulation of Pichinde antigens due primarily to phagocytosis of virus released from sites of synthesis in other tissues.

Respectfully submitted,

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